

1393.002
2302-1393
PATENT

- 1 -

CERTIFICATE OF MAILING BY "EXPRESS MAIL"
"Express Mail" Mailing Label No. EM 336 912 016 45
Date of Deposit March 18, 1998

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231

PATRICIA K. HERNANDEZ
(Typed or Printed Name of Person Mailing Paper or Fee)

Patricia K. Hernandez
(Signature of Person Mailing Paper or Fee)

5 **DETOXIFIED MUTANTS OF BACTERIAL ADP-RIBOSYLATING
 TOXINS AS PARENTERAL ADJUVANTS**

Cross-Reference to Related Application

 This application is related to provisional
10 patent application serial no. 60/041,227, filed March 21,
 1997, from which priority is claimed under 35 USC
 §119(e) (1) and which is incorporated herein by reference
 in its entirety.

15 Field of the Invention

 The present invention relates to adjuvants
 useful for the administration of antigens to organisms.
 In particular, the adjuvants of the invention allow the
 parenteral delivery of vaccines to raise an immune
20 response.

Background of the Invention

 Advances in recombinant DNA technology have
 made possible the generation of a variety of vaccines,
25 such as subunit vaccines and DNA-based vaccines. These
 are in addition to the more traditional killed or
 attenuated vaccines. Adjuvants that enhance the immune
 system's response to antigenic material are known;
 however, currently, few adjuvants are approved for human
30 usage, although additional adjuvants are in pre-clinical
 and clinical studies.

 The ADP-ribosylating bacterial toxins, a group
 of potent toxins to humans, include diphtheria toxin,
 pertussis toxin (PT), cholera toxin (CT), the *E. coli*
35 heat-labile toxins (LT1 and LT2), *Pseudomonas* endotoxin

09044696-031898
3687E0-96944060

5 A, *C. botulinum* C2 and C3 toxins, as well as toxins from
C. perfringens, *C. spiriforma* and *C. difficile*. These
toxins are composed of a monomeric, enzymatically active
A subunit which is responsible for ADP-ribosylation of
GTP-binding proteins, and a non-toxic B subunit which
binds receptors on the surface of the target cell and
delivers the A subunit across the cell membrane.

10 In the case of CT and LT, the A subunit is
known to increase intracellular cAMP levels in target
cells, while the B subunit is pentameric and binds to GM1
ganglioside receptors. (LT-B also binds additional
receptors.)

15 Previously, observations were made
demonstrating that CT is able to induce mucosal and
systemic immunity against itself when administered
intraduodenally (i.e. to a mucosal surface). The
membrane-binding function of CT was shown to be essential
for mucosal immunogenicity, but cholera toxoid, also
known as the B subunit of CT (CT-B) was inactive in
20 isolation (Pierce and Gowans, *J. Exp. Med.* 1975; 142:
1550; Pierce, *J. Exp. Med.* 1978; 148: 195-206).

25 Subsequently, it was demonstrated that native
CT induced immunity to co-administered antigens (Elson,
Curr. Top. Microbiol. Immunol., 1989; 146:29; Elson and
Ealading, *J. Immunol.* 1984; 133:2892-2897; Elson and
Ealading, *Ibid.* 1984; 132:2736-2741; Elson et al., *J.*
Immunol. Meth. 1984; 67:101-118; Lycke and Homgren,
Immunology 1986; 59:301-339) and that immune responses
may be elicited to diphtheria or tetanus toxoids when these
30 antigens are applied to skin in combination with CT.

Two approaches have been taken in order to
address the problem of CT toxicity. The first approach
has involved the use of CT-B as a mucosal adjuvant. CT-B
is entirely non-toxic. An adjuvant effect for co-
35 administered CT-B has been alleged in a number of

00044696 0399
RECEIVED 96944060

publications (Tamura et al., *J. Immunol.* 1992; 149:981-988; Hirabayashi et al., *Immunology* 1992; 75: 493-498; Gizurarson et al., *Vaccine* 1991; 9:825-832; Kikuta et al., *Vaccine* 1970; 8:595-599; Hirabayashi et al. *Ibid.* 1990; 8:243-248; Tamura et al., *Ibid.* 1989; 7:314-32-; Tamura et al., *Ibid.* 1989; 7:257-262; Tamura et al., *Ibid.* 1988; 6:409-413; Hirabayashi et al., *Immunology* 1991; 72:329-335 Tamura et al., *Vaccine* 1989; 7:503-505).

However, a number of aspects of the observations reported above were not entirely convincing. For example, it was noted that the adjuvant effect ascribed to CT-B was not H-2 (MHC) restricted. It is known, however, that the immune response to CTB is H-2 (MHC) restricted (Elson and Ealding, *Eur. J. Immuno.* 1987; 17:425-428). Moreover, the alleged adjuvant effect was observed even in individuals already immune to CT-B.

Other groups were unable to observe any mucosal adjuvant effect attributable to CT-B (Lycke and Holmgren, *Immunology* 1986; 59:301-308; Lycke et al., *Eur. J. Immunol.* 1992: 22:2277-2281). Experiments with recombinant CT-B (Holmgren et al., *Vaccine* 1993; 11:1179-1183) confirmed that the alleged effect is largely, if not exclusively, attributable to low levels of contamination of CT-B preparations with CT.

A second approach to eliminating the toxicity of CT has been to mutate the active holotoxin in order to reduce or eliminate its toxicity. The toxicity of CT resides in the A subunit and a number of mutants to CT and its homologue, LT, comprising point mutations in the A subunit, are known in the art. See, for example, International Patent Application W092/19265. It is accepted in the art that CT and LT are generally interchangeable, showing considerable homology. ADP-ribosylating bacterial toxin mutants have been shown to

0044596 037898

act as mucosal adjuvants, see International Patent Application W095/17211.

Summary of the Invention

5 Accordingly, there remains a need for an active
adjuvant which may be used to increase the immunogenicity
of an antigen when administered parenterally, such as
intramuscularly, subcutaneously, intravenously,
transcutaneously or intradermally. The present invention
10 provides for such parenteral adjuvants in the form of
non-toxic ADP ribosylating bacterial toxins. It is
demonstrated herein that such mutants, lacking toxicity,
are active as parenteral adjuvants and produce high
antibody titers and/or induction of cytotoxic T-
15 lymphocytes (CTLs).

 In one embodiment, then, the subject invention
is directed to a parenteral adjuvant composition
comprising a detoxified mutant of a bacterial ADP-
ribosylating toxin as the parenteral adjuvant and at
20 least one selected antigen.

 In another embodiment, the invention is
directed to a parenteral adjuvant composition comprising
a detoxified mutant of a bacterial ADP-ribosylating toxin
as the parenteral adjuvant and a pharmaceutically
25 acceptable topical vehicle.

 In yet another embodiment, the invention is
directed to a parenteral adjuvant composition comprising
a detoxified mutant of a bacterial ADP-ribosylating toxin
as the parenteral adjuvant, a pharmaceutically acceptable
30 topical vehicle and at least one selected antigen.

 In another embodiment, the invention is
directed to a method for making a parenteral adjuvant
composition comprising combining a detoxified mutant of a
bacterial ADP-ribosylating toxin as the parenteral
35 adjuvant with at least one selected antigen.

5627E0 96944060

In still a further embodiment, the invention is directed to a method of making a parenteral adjuvant composition comprising combining a detoxified mutant of a bacterial ADP-ribosylating toxin as the parenteral
5 adjuvant with a pharmaceutically acceptable topical vehicle.

In another embodiment, the invention is directed to a method for immunizing a vertebrate subject comprising parenterally administering to the vertebrate
10 subject an immunologically effective amount of

a) an adjuvant comprising a detoxified mutant of a bacterial ADP-ribosylating toxin in combination with a pharmaceutically acceptable vehicle; and

b) at least one selected antigen.

In particularly preferred embodiments, the non-toxic adjuvant is a detoxified mutant selected from the group consisting of cholera toxin (CT), pertussis toxin (PT), and an *E. coli* heat-labile toxin (LT), particularly
15 LT-K63, LT-R72, CT-S109, and PT-K9/G129.

20 These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

25 ^{SEQ ID NOS: 1-4}
Figures 1A-1B[^] show the DNA and corresponding amino acid sequences of a wild-type subunit A from an *E. coli* heat labile toxin (LT) (SEQ ID NOS:1 and 2) and a cholera toxin (CT) (SEQ ID NOS:3 and 4).

Figure 2 shows the serum anti-LT antibody
30 response following transcutaneous administration of representative adjuvant compositions of the present invention. Circles indicate titers from individual mice. If less than five circles are visible per group, two or more values were identical and circles were superimposed.

35

Full triangles indicate mean titers per group \pm standard deviation.

unS \triangle
5

Detailed Description of the Invention

5 The practice of the present invention will
employ, unless otherwise indicated, conventional
techniques of molecular biology, microbiology,
recombinant DNA, and immunology, which are within the
skill of the art. Such techniques are explained fully in
10 the literature. See e.g., Sambrook, et al., MOLECULAR
CLONING; A LABORATORY MANUAL, SECOND EDITION (1989); DNA
CLONING, VOLUMES I AND II (D.N Glover ed. 1985);
OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC
ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984);
15 TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins
eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986);
IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B.
Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984);
the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.);
20 GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller
and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory),
METHODS IN ENZYMOLOGY Vol. 154 and Vol. 155 (Wu and
Grossman, and Wu, eds., respectively), Mayer and Walker,
eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR
25 BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN
PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition
(Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IM-
MUNOLOGY, VOLUMES I-IV (D.M. Weir and C. C. Blackwell eds
1986).

30 All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include

plural references unless the content clearly dictates otherwise.

The following amino acid abbreviations are used throughout the text:

5	Alanine (Ala)	A	Arginine (Arg)	R
	Asparagine (Asn)	N	Aspartic acid (Asp)	D
	Cysteine (Cys)	C	Glutamine (Gln)	Q
	Glutamic acid (Glu)	E	Glycine (Gly)	G
10	Histidine (His)	H	Isoleucine (Ile)	I
	Leucine (Leu)	L	Lysine (Lys)	K
	Methionine (Met)	M	Phenylalanine (Phe)	F
	Proline (Pro)	P	Serine (Ser)	S
	Threonine (Thr)	T	Tryptophan (Trp)	W
15	Tyrosine (Tyr)	Y	Valine (Val)	V

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "parenteral" is meant introduction into the body outside of the digestive tract, such as by subcutaneous, intramuscular, transcutaneous, intradermal, or intravenous administration. This is to be contrasted with adjuvants that are delivered to a mucosal surface, such as oral, intranasal, vaginal, or rectal.

As used herein, "detoxified" refers to both completely nontoxic and low residual toxic mutants of the toxin in question. Preferably, the detoxified protein retains a toxicity of less than 0.01% of the naturally occurring toxin counterpart, more preferably less than 0.001% and even more preferable, less than 0.0001% of the toxicity of the naturally occurring toxin counterpart. The toxicity may be measured in mouse CHO cells or preferably by evaluation of the morphological changes

RECEIVED - 9694060

induced in Y1 cells. In particular, Y1 cells are adrenal tumor epithelial cells which become markedly more rounded when treated with a solution containing CT or LT (Ysamure et al., *Cancer Res.* (1966) 26:529-535). The toxicity of
5 CT and LT is correlated with this morphological transition. Thus, the mutant toxins may be incubated with Y1 cells and the morphological changes of the cells assessed.

The term "toxoid" as used herein means a
10 genetically detoxified toxin.

By "antigen" is meant a molecule which contains one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a cellular antigen-specific immune response when the
15 antigen is produced, or a humoral antibody response. Such epitopes may include from about 3 to about 20 or more amino acids. Normally, a B cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T cell epitope, such as a CTL
20 epitope, will include at least about 7-9 amino acids, and a helper T cell epitope at least about 12-20 amino acids. The term denotes both subunit antigens, i.e., antigens which are separate and discrete from a whole organism with which the antigen is associated in nature, as well
25 as killed, attenuated or inactivated bacteria, viruses, parasites or other microbes. Antibodies such as anti-idiotypic antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the
30 definition of antigen as used herein.

For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi. The term also intends any of the various tumor antigens. Furthermore, for purposes
35 of the present invention, an "antigen" refers to a

03469-03469

surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T cells. Helper T cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T cells and/or other white blood cells, including those derived from CD4+ and CD8+ T cells.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376; and the examples below.

Thus, an immunological response as used herein may be one which stimulates the production of CTLs, and/or the production or activation of helper T cells. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an

immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or $\gamma\delta$ T-cells directed specifically to an antigen or
5 antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be
10 determined using standard immunoassays and neutralization assays, well known in the art. For a general overview of the immune system and immunological mechanisms see for example: Janeway, C.A. and Travers, P., IMMUNOBIOLOGY, 2nd ed. 1996, Current Biology Ltd./Garland Publishing,
15 New York, NY.

A composition which contains a selected antigen along with a detoxified mutant of a bacterial ADP-ribosylating toxin of the present invention, or a vaccine composition which is coadministered with the subject
20 adjuvant, displays "enhanced immunogenicity" when it possesses a greater capacity to elicit an immune response than the immune response elicited by an equivalent amount of the antigen administered without the adjuvant. Thus, a vaccine composition may display "enhanced
25 immunogenicity" because the antigen is more strongly immunogenic or because a lower dose or fewer doses of antigen are necessary to achieve an immune response in the subject to which the antigen is administered. Such enhanced immunogenicity can be determined by
30 administering the adjuvant composition and antigen controls to animals and comparing antibody titers and/or cellular-mediated immunity against the two using standard assays such as radioimmunoassay, ELISAs, CTL assays, and the like, well known in the art.

function when administered parenterally, they permit a convenient method of conferring immunity to substances that are not amenable to other modes of administration. Accordingly, the present system is useful with a wide
5 variety of antigens and provides a powerful tool to prevent and/or treat a large number of infections.

Regarding the present invention, any detoxified mutant of a bacterial ADP-ribosylating toxin can be used as a parenteral adjuvant. Such mutants optionally
10 comprise one or more amino acid additions, deletions or substitutions that result in a molecule having reduced toxicity while retaining adjuvanticity. If an amino acid is substituted for the wild-type amino acid, such substitutions may be with a naturally occurring amino
15 acid or may be with a modified or synthetic amino acid. Substitutions which alter the amphotericity and hydrophilicity while retaining the steric effect of the substituting amino acid as far as possible are generally preferred.

20 The mutants of the invention are preferably in the form of a holotoxin, comprising the mutated A subunit and the B subunit, which may be oligomeric, as in the wild-type holotoxin. The B subunit is preferably not mutated. However, it is envisaged that a mutated A
25 subunit may be used in isolation from the B subunit, either in an essentially pure form or complexed with other agents, which may replace the B subunit and/or its functional contribution.

As explained above, in addition to the
30 completely nontoxic ADP-ribosylating bacterial toxins, toxins can be used wherein a residual toxicity greater than 100 to 10,000 fold lower, or more, than its naturally occurring counterparts is found.

Particularly suitable are detoxified mutants of
35 diphtheria toxin, CT, LT, or PT; such mutations are known

030456-01892

in the art. For example, particular mutant LTs in accordance with the invention may possess the following mutations of the A subunit: a Val to Asp, Glu or Tyr substitution at position 53; a Ser to Lys substitution at position 63 (termed LT-K63 herein); an Ala to Arg substitution at position 72 (termed LT-R72 herein); a Val to Lys or Tyr substitution at position 97; a Tyr to Lys, Asp or Ser substitution at position 104; a Pro to Ser substitution at position 106; an Arg to Gly substitution at position 192.

Since the amino acid sequences of CT-A and LT-A are substantially conserved (see Figures 1A-1B, (SEQ ID NOS:1-4)), the changes described above with respect to LT can also be made to the corresponding positions in CT. A particularly preferred CT mutant comprises a substitution of Ser at position 109 (termed CT-S109 herein).

A preferred detoxified mutant of *Bordetella pertussis* is a double mutant where Lys replaces Arg at amino acid position 9 and Gly replaces Glu at amino acid position 129 (termed PT-K9/G129 herein). Many other suitable pertussis toxin (PT) mutants are known in the art.

Methods for the design and production of mutants of CT and/or LT are known in the art. Suitable methods are described in International Patent application W093/13202, as well as W092/19265. In particular, such mutant toxins may be synthesized chemically, using conventional peptide synthesis techniques. See, e.g., See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of*

Peptide Synthesis, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology, supra*, Vol. 1, for classical solution synthesis.

5 Alternatively, and preferably, mutations can be made to the wild-type sequence using conventional recombinant techniques such as by preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding the wild-type
10 protein using restriction endonuclease digestion. (See, e.g., Kunkel, T.A. *Proc. Natl. Acad. Sci. USA* (1985) 82:448; Geisselsoder et al. *BioTechniques* (1987) 5:786.) Alternatively, the mutations can be effected using a mismatched primer which hybridizes to the wild-type
15 nucleotide sequence (generally cDNA corresponding to the RNA sequence), at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by
20 keeping the mutant base centrally located. Zoller and Smith, *Methods Enzymol.* (1983) 100:468. Primer extension is effected using DNA polymerase, the product cloned and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Selection can
25 be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations. See, e.g., Dalbie-McFarland et al. *Proc. Natl. Acad. Sci USA* (1982) 79:6409. PCR mutagenesis will also find use for
30 effecting the desired mutations.

 The adjuvant of the invention is preferably administered in admixture with at least one antigen against which it is desired to raise an immune response. If the antigen and the adjuvant are not in admixture, it
35 is preferred that they be administered within a

ORIGINAL DOCUMENT

relatively short time of each other, at the same site of administration, although there may be a delay of up to 5 days and a two-injection site regime. Thus, the adjuvant may be administered prior or subsequent to, or concurrent with the selected antigen. It has been observed that the adjuvant effect provided by wild-type CT is short-lived (see Lycke and Homgren, *Immunology* 1986: 59: 301-308).

In an alternative embodiment, the adjuvant of the present invention may be administered, optionally in isolation from other antigens, as a boost following systemic or mucosal administration of a vaccine.

Diseases against which the subject may be immunized include all diseases capable of being treated or prevented by immunization, including viral diseases, allergic manifestations, diseases caused by bacterial or other pathogens, such as parasitic organisms, AIDS, autoimmune diseases such as Systemic Lupus Erythematosus, Alzheimer's disease and cancers. Vaccine formulations suitable for delivery may be prepared as set out hereinbelow, while further formulations will be apparent to those of skill in the art.

Thus, the antigen may be any antigen to which it is desired to raise an immune response in the subject. Suitable antigens comprise bacterial, viral, fungal and protozoan antigens derived from pathogenic organisms, as well as allergens, and antigens derived from tumors and self-antigens. Typically, the antigen will be a protein, polypeptide or peptide antigen, but alternative antigenic structures, such as nucleic acid antigens, carbohydrate antigens and whole or attenuated or inactivated organisms such as bacteria, viruses or protozoa are included.

Specific examples of antigens useful in the present invention include a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1

RECEIVED - 031990

and HSV-2 glycoproteins gB, gD and gH; antigens derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7. (See, e.g. Chee et al., *Cytomegaloviruses* (J.K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et al., *J. Gen. Virol.* (1988) 69:1531-1574, for a discussion of the various HSV-1 encoded proteins; U.S. Patent No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., *Nature* (1984) 310:207-211, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, *J. Gen. Virol.* (1986) 67:1759-1816, for a review of VZV.)

The adjuvant compositions of the present invention can also be used to deliver antigens from the hepatitis family of viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV). By way of example, the viral sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including E1 (also known as E) and E2 (also known as E2/NSI). (See, Houghton et al., *Hepatology* (1991) 14:381-388, for a discussion of HCV proteins, including E1 and E2.) The sequences of these proteins, as well as antigenic fragments thereof, will find use in the present methods. Similarly, the sequence for the δ -antigen from HDV is known (see, e.g., U.S. Patent No. 5,378,814) and this antigen can also be conveniently used in the present methods.

Antigens derived from other viruses will also find use in the claimed methods, such as without limitation, proteins from members of the families Picornaviridae (e.g., polioviruses, etc.); Caliciviridae; 5 Togaviridae (e.g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabdoviridae (e.g., rabies virus, etc.); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae 10 (e.g., influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviridae (e.g., HTLV-I; HTLV-II; HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV_{IIIB}, HIV_{SF2}, HIV_{LAV}, HIV_{LAI}, HIV_{MN}; HIV-1_{CM235}, 15 HIV-1_{US4}; HIV-2; simian immunodeficiency virus (SIV) among others. See, e.g. Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991), for a description of these and other viruses.

20 For example, the present adjuvants can be used in conjunction with the gp120 envelope protein from HIV_{SF2}, HIV-1_{CM235}, HIV-1_{US4}, HIV-1_{IIIB} and HIV-1_{LAI}. The gp120 sequences for these and a multitude of additional HIV-1 and HIV-2 isolates, including members of the various 25 genetic subtypes of HIV, are known and reported (see, e.g., Myers et al., Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico (1992); Myers et al., *Human Retroviruses and Aids*, 1990, Los Alamos, New Mexico: Los Alamos National Laboratory; and Modrow et 30 al., *J. Virol.* (1987) 61:570-578, for a comparison of the envelope sequences of a variety of HIV isolates) and sequences derived from any of these isolates will find use in the present methods. Furthermore, the invention is equally applicable to other immunogenic proteins 35 derived from any of the various HIV isolates, including

00456996-9694060

any of the various envelope proteins such as gp160 and gp41, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol region.

Additionally, the envelope glycoproteins HA and
5 NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology* (1990) 179:759-767; Webster et al., "Antigenic variation among type A influenza viruses," p. 127-168.
10 In: P. Palese and D.W. Kingsbury (ed.), *Genetics of influenza viruses*. Springer-Verlag, New York). Thus, proteins derived from any of these isolates can also be used in the techniques described herein.

The compositions and methods described herein
15 will also find use with numerous bacterial antigens, such as those derived from organisms that cause diphtheria, cholera, tuberculosis, tetanus, pertussis, meningitis, and other pathogenic states, including, without limitation, *Bordetella pertussis*, *Neisseria meningitides*
20 (A, B, C, Y), *Hemophilus influenza* type B (HIB), and *Helicobacter pylori*. Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

Furthermore, the methods and compositions
25 described herein provide a means for treating a variety of malignant cancers. For example, the system of the present invention can be used to mount both humoral and cell-mediated immune responses to particular proteins specific to the cancer in question, such as an activated
30 oncogene, a fetal antigen, or an activation marker. Such tumor antigens include any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc. (Boon, T. *Scientific American* (March 1993):82-89); any of the various tyrosinases; MART 1 (melanoma antigen
35 recognized by T cells), mutant ras; mutant p53; p97

2025 RELEASE UNDER E.O. 14176

removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of non-specifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen.

- 5 B cells, expressing membrane-bound immunoglobulin specific for the antigen, will bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybrid-
- 10 omas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not
- 15 bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice). See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980);
- 20 Hammerling et al., *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal
- 25 antibodies produced against the hormone of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc.

Compositions according to the invention may comprise one or more antigens. Furthermore, one or more

30 "pharmaceutically acceptable excipients or vehicles" are present, such as water, saline, glycerol, polyethyleneglycol, hyaluronic acid, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the

35 like, may be present in such vehicles.

000446-031999

emulsifier and the aqueous phase. The adjuvant, and optionally the antigen, can be included in any one of the phases, or added to the formed emulsion. The oil phase is typically comprised of petrolatum with one or more
5 higher molecular weight alcohols such as cetyl or steryl alcohol. The aqueous phase generally contains preservatives, the water-soluble components of the emulsion system, humectants (such as glycerin, propylene glycol or a polyethylene glycol), as well as optional
10 stabilizers, antioxidants, buffers and the like.

The above pharmaceutical ointments are formed by dispersing finely divided or dissolved adjuvant and, optionally one or more selected antigens, uniformly throughout the vehicle or base. Creams, lotions and
15 emulsions can be formed by way of a two-phase heat system, wherein oil-phase ingredients are combined under heat to provide a liquified, uniform system. The aqueous-phase ingredients are separately combined using heat. The oil and aqueous phases are then added together
20 with constant agitation and allowed to cool. At this point, concentrated agents may be added as a slurry. Volatile or aromatic materials can be added after the emulsion has sufficiently cooled. Preparation of such pharmaceutical compositions is within the general skill
25 of the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

The adjuvants can also be incorporated into gel formulations using a two-phase gel system. Such systems
30 generally comprise a suspension or network of small, discrete particles interpenetrated by a liquid to provide a dispersed phase and a liquid phase. Single-phase gel systems are formed by distributing organic macromolecules uniformly throughout a liquid such that there are no
35 apparent boundaries between the dispersed and liquid

RECEIVED SEP 14 1990

phases. Suitable gelling agents for use herein include synthetic macromolecules (e.g., Carbomers®, polyvinyl alcohols and polyoxyethylene-polyoxypropylene copolymers), gums such as tragacanth, as well as sodium
5 alginate, gelatin, methylcellulose, sodium carboxymethylcellulose, methylhydroxyethyl cellulose and hydroxyethyl cellulose. In order to prepare a uniform gel, dispersing agents such as alcohol or glycerin can be added, or the gelling agent can be dispersed by
10 trituration, mechanical mixing or stirring, or combinations thereof.

Lotion preparations are generally liquid or semiliquid preparations containing the adjuvant and, optionally, one or more selected antigens, in a suitable
15 vehicle. Lotions are formed by suspending finely divided solids in an aqueous medium. Optional dispersing agents can be employed to aid in the preparation of the liquid formulation, as well as one or more surface-active agents.

20 In the cream and ointment formulations described hereinabove, optional ingredients can include materials such as antioxidants, viscosity modifiers (e.g., paraffin wax or lanolin wax), and topical absorption rate modifiers. Actual methods of preparing
25 any of the above formulations are known, or will be apparent, to those skilled in the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

Immunogenic compositions used as vaccines
30 comprise an immunologically effective amount of the adjuvant and an antigen, as well as any other the above-mentioned components, as needed. By "immunologically effective amount", is meant that the administration of that amount to an individual, either in a single dose or
35 as part of a series, is such that an immune response can

00495-03990

be generated in the subject to which it is administered. The exact amount necessary will vary depending on the subject being treated; the age and general condition of the subject to be treated; the capacity of the subject's immune system to synthesize antibodies and/or mount a cell-mediated immune response; the degree of protection desired; the severity of the condition being treated; the particular antigen selected and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. Thus, an "immunologically effective amount" will fall in a relatively broad range that can be determined through routine trials. In general, an amount on the order of about 0.1 μ g to about 1000 μ g, more preferably about 1 μ g to about 100 μ g.

Similarly, the adjuvant will be present in an amount such that the antigen displays "enhanced immunogenicity," as defined above, as compared to administration of the antigen alone, without the adjuvant. Amounts which are effective for eliciting an enhanced immune response can be readily determined by one of skill in the art.

Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

Additional adjuvants can be used to enhance effectiveness; such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO

90/14837), containing 5% Squalene, 0.5% Tween 80, and
0.5% Span 85 (optionally containing various amounts of
MTP-PE (see below), although not required) formulated
into submicron particles using a microfluidizer such as
5 Model 110Y microfluidizer (Microfluidics, Newton, KA),
(b) SAF, containing 10%- Squalane, 0.4% Tween 80, 5%
pluronic-blocked polymer L121, and thr-MDP (see below)
either microfluidized into a submicron emulsion or
vortexed to generate a larger particle size emulsion, and
10 (c) Ribⁱ™ adjuvant system (RAS), (Ribi Immunochem,
Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and
one or more bacterial cell wall components from the group
consisting of monophosphorylipid A (MPL), trehalose
dimycolate (TDM), and cell wall skeleton (CWS),
15 preferably MPL + CWS (Detox™); (3) saponin adjuvants,
such as Stimulon™ (Cambridge Bioscience, Worcester, MA)
may be used or particles generated therefrom such as
ISCOMs (immunostimulating complexes); (4) Complete
Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant
20 (IFA); (5) cytokines, such as interleukins (e.g., IL-1,
IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons
(e.g., gamma interferon), macrophage colony stimulating
factor (M-CSF), tumor necrosis factor (TNF), etc; and (6)
other substances that act as immunostimulating agents to
25 enhance the effectiveness of the composition. Alum and
MF59 are preferred. Muramyl peptides include, but are
not limited to, N-acetyl-muramyl-L-threonyl-Disoglutamine
(thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine
(nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-
30 alanine-2- (1'-2'-dipalmitoyl-sn-glycero-3 -
huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

The invention further provides a method for the
manufacture of an adjuvanted vaccine comprising the steps
of:

a) performing site-directed mutagenesis on the A subunit of a bacterial ADP-ribosylating toxin in order to detoxify the toxin; and

b) combining the detoxified toxin with an
5 antigen, such that it functions as a parenteral adjuvant.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are
10 offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures,
15 etc.), but some experimental error and deviation should, of course, be allowed for.

20

25

30

35

Example 1

Production of LT-K63

LT-K63, for use in the following experiments, was made as follows. Site-directed mutagenesis was used to replace the Ser residue at position 63 of the A subunit of LT with Lys in order to construct a non-toxic LT mutant that would still assemble as a holotoxin with cell binding activity. The mutant protein, named LT-K63, was purified and tested for ADP-ribosyltransferase and toxic activity in several assays. LT-K63 was still able to bind GM1 ganglioside receptor but showed a complete loss of enzymatic activity, in agreement with published data (Lobet et al., *Infect. Immun.* 1991; 59:2870-2879). Further, LT-K63 was inactive in the ~~mouse~~^{rabbit} ileal loop assay and *in vitro* on Y1 cells.

Example 2

Parenteral adjuvant activity of LT-K63
with HSV gD2 in mice

20 The LT-K63 mutant, produced as described in
Example 1, was tested as a parenteral adjuvant with
herpes simplex virus-type 2 (HSV-2) gD antigen as
follows.

a. Mice were immunized twice by intramuscular injection one month apart with a 10 μ g dose of the LT-K63 mutant and a 10 μ g dose of HSV-2 gD2 antigen. Sera were collected on day 0 and two weeks after the second immunization (day 42). The antibody responses against HSV-2 gD2 were measured by ELISA. The geometric mean titers plus or minus standard error are listed in Table 1. This experiment illustrates the ability of the LT-K63 mutant in combination with HSV-2 gD2 to produce an immune response in mice.

Table 1. Serum anti-gD2 titers of mice immunized with LT-K63 and gD2		
Animal #	Day 0	Day 42
BL900	<10	4510
BL899	<10	45920
BL900	<10	7535
BL901	<10	56585
BL902	<10	74085
BL903	<10	8845
BL904	<10	4340
BL905	<10	19430
BL906	<10	6380
BL907	<10	9125
GMT +/- SEM		13975 +/- 4726

b. Mice were immunized twice by intramuscular injection one month apart with 10 μ g HSV-2 gD2. Sera were collected on day 0 and two weeks after the second immunization. The antibody responses against HSV-2 gD2 were measured by ELISA and are shown as geometric mean titer plus or minus standard error in Table 2. The 90-fold lower antibody response produced by HSV-2 gD2 (150) compared to HSV-2 gD2 combined with LT-K63 (13980) illustrates the parenteral adjuvant activity of LT-K63 with HSV-2 gD2.

Table 2. Serum anti-HSV gD2 antibody titers of mice immunized with gD2		
Animal #	Day 14 Sera	Day 42 Sera
BC048	<10	348
BC048	<10	11
BC049	<10	17
BC050	<10	222
BC051	<10	73
BC050	<10	44
BC053	<10	2053
BC054	<10	2882
GMT +/- SEM		151 +/- 111

Example 3

Parenteral adjuvant activity of LT-K63 with influenza HA in mice

Mice were immunized twice by intramuscular injection one month apart with 1 μ g LT-K63 (produced as described in Example 1) and 1 μ g A/Texas HA (hemagglutinin) antigen or 1 μ g A/Texas HA alone. Sera were collected two weeks after the second immunization. The anti-HA ELISA titers are shown as geometric mean titer plus or minus standard error in Table 3. The 11-fold higher antibody response observed in the group receiving HA antigen combined with the LT-K63 mutant (70380) compared with the group receiving HA antigen alone (6390) illustrates the effectiveness of LT-K63 as a parenteral adjuvant with influenza HA antigen.

Table 3. Serum anti-HA titers of mice immunized with HA or HA with LT-K63

HA Animal #	Day 42		HA + LT-K63 Animal #	Day 42
CN 622	3698			
CN 623	7778			
CN 624	5506			
CN 625	5142			
CN 626	7109			
CN 627	7422			
CN 629	51463			
CN 629	19299			
CN 630	2906			
CN 631	427			
CN 632	2601		CN 642	73486
CN 633	4817		CN 643	70019
CN 634	7315		CN 644	43773
CN 635	19056		CN 645	79454
CN 636	19979		CN 646	229580
CN 637	2049		CN 647	43157
CN 638	3404		CN 648	29928
CN 639	12447		CN 649	84437
CN 640	4817		CN 650	88956
CN 641	16752		CN 651	74790
GMT+/-SEM	6391+/-1484			70378+/-12194

Example 4

Parenteral adjuvant activity of LT-K63
with HIV p24 gag in mice

a. Mice were immunized three times by subcutaneous injection 1 week apart with 10 μ g LT-K63 (produced as described in Example 1) and 10 μ g HIV p24

gag or 10 μ g HIV p24 gag alone. HIV p24 gag-specific CTL activity is depicted in Table 4. CTL activity was measured in a standard chromium release assay and is presented as % specific lysis. In particular, SVBalb (H-2d) and MC57 (H-2b) target cells were incubated with 51Cr and 10⁶ p7g peptide for 1 hour. Effector cells (E) were cultured with target cells (T) at various E:T ratios for 4 hours. The average cpm from duplicate wells was used to calculate percent specific 51Cr release. Allogeneic Mc57 target cells and syngeneic SvBalb target cells had low background killing. Syngeneic SvBalb cells pulsed with HIV p24 gag epitope peptide p7g had 69% specific lysis at a 50:1 E:T ratio for HIV p24 gag with LT-K63. In contrast, HIV p24 gag alone induced only 29% killing under the same conditions. The group receiving LT-K63 had a higher CTL response in contrast to the gag alone group. This illustrates the adjuvant activity of LT-K63 for CTL induction with HIV p24 gag.

Table 4. CTL Responses of Mice Immunized with HIV gag and LT-K63K				
	%specific lysis			
	Effector: Target	Mc57/p7g	SvB/p7g	SvB/-
	50:1			
LT-K63 + HIV p24 gag	10	2	69	7
	10	3	31	7
	2	4	14	4
HIV p24 gag	50:1	5	29	4
	10	3	12	2
	2	2	5	3

b. Mice were immunized twice by subcutaneous injection one month apart with 10 μ g LT-K63 and 10 μ g HIV

p24 gag. Sera were collected two weeks after the second immunization. The anti-HIV p24 gag titers are shown as geometric mean titer plus or minus standard error in Table 5. This experiment illustrates the ability of LT-K63 in combination with HIV p24 gag to produce an anti-HIV p24 gag response in mice.

10

15

Table 5. Antibody responses of mice immunized with p24 gag		
Animal #	Day 0	Day 42
CP386	13	220406
CP387	15	153674
CP388	9	235706
CP389	20	350167
GMT+/-SEM		229900+/-38800

Example 5

20

Transcutaneous Adjuvant Activity of LT-K63 and LT-R72

25

30

For the following experiments, the LT wild-type (LTwt), LT-K63 and LT-R72 mutants were obtained as described (Giuliani et al., "Mucosal Adjuvanticity of LTR72, a Novel Mutant of *Escherichia coli* Heat-Labile Enterotoxin with Partial Knock-Out of ADP Ribosyltransferase Activity," *J. Exp. Med.* 187:in press). The antigen used, CRM₁₉₇, is a well-characterized non-toxic diphtheria toxin mutant. See, e.g., Bixler et al. (1989) *Adv. Exp. Med. Biol.* 251:175, Constantino et al. (1992) *Vaccine*; International Publication No. WO 96/14086.

35

For transcutaneous immunization, on day 0, groups of 5 female BALB/c mice were anesthetized with an intraperitoneal injection of 100 μ l/10 g of weight of a solution of Ketavet™ 50 (20% v/v), Rompun™ (3% v/v), and

SECRETED 9604060

Combelen™ (3% v/v) in sterile saline. Mice were then shaved on the back (about 2 cm²), and 100 µl of phosphate-buffered saline (PBS) containing 100 µg of CRM197 and 50 µg of LTwt or LT mutants were gently applied on the shaved skin. Mice were kept under anesthesia for about 1 hour, then washed with lukewarm tap water, and dried. The same procedure was repeated on day 21. Third and fourth immunizations were performed on day 51 and day 66, respectively. On the same dates, control groups of 5 mice received CRM197 (10 µg) and LTwt (1 µg) intranasally (20 µl volume).

Serum samples were taken at days -1, 20, 35, 65, and 80. Antibodies to LT and CRM were determined by standard ELISA procedures.

No anti-CRM antibody response was detectable. As shown in Table 6 and Figure 2, transcutaneous immunization induced a very strong anti-LT antibody response after one immunization (see Table 6), which was boosted after the second immunization (see Table 6 and Figure 2). Thus, transcutaneous immunization (i.e., application of soluble antigens plus mucosal adjuvants on the skin) induced the production of specific antibodies, showing that the immune system responded to the LT proteins. This result evidences that these proteins may be useful as transcutaneous adjuvants.

30

35

2025-03-18 09:44:56

Table 6. Serum anti-LT antibody titers in BALB/c mice immunized transcutaneously							
	mouse n.	day -1 (preimmune)	day 20 (post-1)	day 35 (post-2)	log titer day 35	mean	SD
Group 1 tc LT w.t.	1	0	60856	81127	4.91	5.34	0.37
	2	0	31319	833666	5.92		
	3	0	109229	256225	5.41		
	4	0	129280	182907	5.26		
	5	0	35628	156077	5.19		
Group 2 tc CRM	6	0	0	0			
	7	0	0	0			
	8	0	0	0			
	9	0	0	0			
	10	0	0	0			
Group 3 tc CRM + LT w.t.	11	0	9593	99577	5.00	4.98	0.31
	12	0	4606	73229	4.86		
	13	0	3455	60058	4.78		
	14	0	5137	56589	4.75		
	15	0	20997	327216	5.51		
Group 4 tc CRM + LTK63	16	0	7691	16501	4.22	3.90	0.49
	17	0	6307	37822	4.58		
	18	0	404	2770	3.44		
	19	0	572	5382	3.73		
	20	0	843	3278	3.52		

→

Table 6. Serum anti-LT antibody titers in BALB/c mice immunized transcutaneously							
	mouse n.	day -1 (preimmune)	day 20 (post-1)	day 35 (post-2)	log titer day 35	mean	SD
Group 5 tc CRM + LTR72	21	0	2401	25676	4.41	4.67	0.30
	22	0	6868	45181	4.65		
	23	0	6868	33891	4.53		
	24	0	8049	38174	4.58		
	25	0	19452	186017	5.19		
Group 6 i.n. CRM + LT w.t.	26	0	169516	1195152	6.08	6.17	0.37
	27	0	104288	489685	5.69		
	28	0	210832	4000000	6.60		
	29	0	187184	989957	6.00		
	30	0	289546	3105000	6.49		

*0=negative (titer<50)

Groups 1 to 5 were immunized transcutaneously (tc), group 6 intranasally. For tc immunizations (days 0 and 21), groups of BALB/c mice were shaved on the back (about 2 cm²) and kept under anesthesia for 1 hour. During this time, 100 microliters of PBS containing antigen CRM197 (100 micrograms) and LT or LT mutants (50 micrograms) were **applied on the shaved skin**. Mice were then extensively washed with lukewarm water to avoid possible oral intake of residual antigens. Serum samples were taken at the dates indicated, and tested by ELISA for quantitation of specific antibodies.

-37-~~12~~

Thus, parenteral adjuvants comprising
detoxified mutants of a bacterial ADP-ribosylating toxin
are disclosed. Although preferred embodiments of the
subject invention have been described in some detail, it
5 is understood that obvious variations can be made without
departing from the spirit and the scope of the invention
as defined by the appended claims.

10

15

20

25

30

35

2025 RELEASE UNDER E.O. 14176